

Amendments to the Specification:

Please delete the paragraphs on page 15, lines 4-7 and 8-10 and replace with the following amended paragraphs:

B) Separation of the PEGylated products by size exclusion chromatography ("SEC"). SEC of random 40kDa PEGylated IGFBP-4 was performed on ~~Superose~~ SUPEROSE 6 (cross linked agarose size exclusion media) (Pharmacia) in 20 mM Phosphate pH7.5, 500mM NaCl, flow rate 0.5ml/min.

C) Analysis of PEGylated products by SDS PAGE. Std = ~~Mark~~MARK12 Molecular weight standard (Invitrogen); 3 = polyPEG40-IGFBP-4; 4 = unPEGylated IGFBP-4; 5 = monoPEG40-IGFBP-4.

Please delete the paragraph on page 16, lines 13-25 and replace with the following amended paragraph:

The binding abilities of IGFBP-4 or PEGylated isoforms thereof were determined by a size exclusion chromatography based assay. 70nmol (6ug) of IGF-I are injected on the column (HRP 75, Pharmacia; running conditions: 20mM sodium phosphate pH 7.4, 500mM NaCl, 1ml/min) either alone or together with 96nmol mono20kDa-PEG-IGFBP-4 (equivalent to 25ug wildtype IGFBP-4) after a preincubation step (30min at room temperature). Free IGF-I is quantified by integrating the IGF-I peak of the chromatogram (~~Chromeleon~~ CHROMELEON chromatography management system, Dionex). The peak area is negatively correlated with the binding capacity of IGFBP-4. In the demonstrated experiment, more than 90% of IGF-I is bound by mono20kDa-PEG-IGFBP-4. Similar results were obtained with mono40kDa-PEG-IGFBP-4.

Please delete the paragraph on page 18, lines 22-32 and replace with the following amended paragraph:

The naturation sample was stocked up to 25% $(\text{NH}_4)_2\text{SO}_4$ and centrifuged. The supernatant was dialyzed against 50mM sodium citrate, 100mM NaCl (pH 4.5) and brought to 0.8M $(\text{NH}_4)_2\text{SO}_4$ and 0.2M arginine (by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ and dilution of a 1M arginine/HCl stock solution). After pH adjustment to pH 8.5 with NaOH, the sample was applied to a ~~phenyl-sepharose~~ PHENYL SEPHAROSE (phenyl-substituted cross-linked agarose hydrophobic interaction chromatography medium) column (~~phenyl-sepharose~~ PHENYL SEPHAROSE fast flow (Pharmacia); equilibrated with 20 mM sodium phosphate, 100mM NaCl, 1M $(\text{NH}_4)_2\text{SO}_4$ (pH 7.5)). The column was washed with equilibration buffer without $(\text{NH}_4)_2\text{SO}_4$. Elution of IGFBP-4 was achieved in a gradient from 20mM sodium phosphate to 20 mM sodium phosphate supplemented with 50% ethylene glycol and a post-elution wash with 20mM sodium phosphate, 100mM, NaCl, 50% ethylene glycol, pH 7.5.

Please delete the paragraph on page 19, lines 1-5 and replace with the following amended paragraph:

The eluate was pooled according to SDS-PAGE, diluted 1:2 WITH 50mM citrate pH 4.2 and applied on a ~~S-sepharose~~ S-SEPHAROSE (crosslinked agarose cation exchange chromatography media) column (Pharmacia). The column was washed with 20mM sodium phosphate pH 7.5 and elution was performed with a gradient to 20mM sodium phosphate, 600mM NaCl. IGFBP-4 was finally pooled on the basis of SDS-PAGE.

Please delete the paragraph on page 21, lines 18-21 and replace with the following amended paragraph:

Preparative separation of PEGylation products for biochemical and biological analysis is achieved by size exclusion chromatography on a ~~sephacryl~~-SEPHACRYL S 400 column

(Pharmacia) in a running buffer consisting of 20mM sodium phosphate pH 7.5 supplemented with 500mM sodium chloride.

Please delete the paragraph on page 23, lines 2-7 and replace with the following amended paragraph:

Preparative separation of PEGylation products for biochemical and biological analysis was achieved by size exclusion chromatography on a ~~sephaeryl~~ SEPHACRYL S 300 column (cross-linked dextran polyacrylamide) (Pharmacia) in a running buffer consisting of 20mM sodium phosphate pH 7.5 supplemented with 500mM sodium chloride. The 20kDa PEGylated species elute earlier in size exclusion chromatography (SEC) as compared to the unmodified form. This is due to an increased hydrodynamic radius of the molecule.

Please delete the paragraph on page 23, lines 15-24 and replace with the following amended paragraph:

Residual PEGylation reagents that did not react with IGFBP-4 were removed by ion exchange chromatography (IEC) using a SP ~~sepharose~~ SEPHAROSE (crosslinked agarose cation exchange chromatography media) (Pharmacia). Samples were dialyzed before loading onto the column against 20mM sodium phosphate pH 5.5 to reduce the concentration of sodium chloride and to adjust to the acidic pH. Under these conditions, free PEG did not bind to the column resin and was detected in the column flow through by a colorimetric assay as described by Nag, A., et al. Anal. Biochem. 237 (1996) 224-231. Elution of bound protein was performed in a single step with 300mM sodium chloride in 20mM sodium phosphate pH 5.5. Samples were dialyzed against 20mM sodium phosphate pH 7.5, 150mM sodium chloride before storage or further analysis.

Please delete the paragraph on page 24, lines 17-20 and replace with the following amended paragraph:

All measurements were performed on a ~~Confocor~~ CONFOCOR I (Zeiss, Jena) fluorescence correlation microscope at a wavelength of 543 nm in a buffer consisting of: 100mM HEPES (pH 7.6), 120mM NaCl, 5mM KCl, 1.2mM Mg₂SO₄; 1mM EDTA, 10mM D(+) Glucose, 15mM sodium acetate, 1% dialyzed bovine serum albumin.

Please delete the paragraph on pages 24, lines 27-29 and page 25, lines 1-10 and replace with the following amended paragraph:

Inhibitory constants (IC₅₀ values) for wildtype IGFBP-4 and several PEGylated isoforms were determined in ~~Biacore~~ BIACORE surface plasmon resonance experiments (<http://www.biacore.com>). Briefly, wildtype IGFBP-4 was immobilized to a ~~Biacore~~ BIACORE CM5 chip surface by NHS-EDC coupling chemistry as known from the art (<http://www.biacore.com>). All IGF-I binding experiments were conducted in a commercially available buffer (~~Biacore~~ BIACORE) HBP-EP; 0.01M Hepes pH 7.4; 0.15M NaCl; 3mM EDTA; 0.005% polysorbate 20 (v/v). To determine IC₅₀ values, 10nM IGF-I was mixed with eight concentrations from 0.5 to 1000nM to wildtype IGFBP-4 (or of mono20kDa-PEG-IGFBP-4 or mono40kDa-PEG-IGFBP-4) and applied on the chip with immobilized IGFBP-4. Inhibition was measured as a decrease of response units compared to samples of 10nM pure IGF-I in the absence of IGFBP-4. Mono20kDa-PEG-IGFBP-4 and mono40kDa-PEG-IGFBP-4 inhibited IGF-I binding as efficient as wild type control IGFBP-4 with IC₅₀ values of about 4+/-2nM.